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14. ABSTRACT A cytoplasmic ER mutant (cER), lacking its NLS domain, is completely resistant to ICI-mediated degradation. The inability of ICI to degrade cER is not due to an inability of cER to bind ICI. Furthermore, the resistance phenotype is independent of both length of treatment with ligand and dose of ICI that is administered. The NLS domain may serve as a region of interaction with other proteins that assist in the degradation process. If this statement is true, it is unlikely that keratin 18 (K18), a protein previously reported to be critical for ICI-mediated degradation of ER, is one of these interacting proteins. It appears that K18 can interact with both full-length wild-type ER and with cER lacking the NLS. Therefore, the inability of ICI to degrade cER is not likely due to an inability of cER to interact with K18.

15. SUBJECT TERMS
ER-estrogen receptor alpha ; cER-cytoplasmic ER mutant ; rhER-membrane-tethered ER mutant; ICI-ICI182780 ; E2-estradiol ; NLS-nuclear localization signal

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INTRODUCTION

Selective estrogen receptor down-regulators (SERDs), such as ICI 182780 (ICI, Faslodex), are thought to exert their effects by binding and degrading estrogen receptor alpha (ER); however, the mechanism of degradation is unknown. Our laboratory has shown that a cytoplasmic ER mutant (cER) that lacks a nuclear localization signal (NLS) is completely resistant to ICI-mediated degradation in C4-12 cells. However, a rhodopsin-fused full-length ER (containing its NLS) that is sequestered at the plasma membrane is degraded upon treatment with ICI in this cell line. **I hypothesize, therefore, that the NLS domain itself, and not merely the subcellular localization, is the critical determinant in ICI-mediated degradation of ER**. I predict that the NLS serves as a region for interactions with other proteins that may assist in the degradation process.

BODY

1) Research Training Environment

The Breast Center at Baylor College of Medicine (BCM) provides a unique training environment with multiple opportunities for me to grow as a young research scientist. In the past year, I have taken full advantage of these opportunities and will outline my primary accomplishments here.

- attended and presented data in poster format at the Breast Center/Cancer Center retreat (November 2007)
- completed and received a grade of 91.5% in the Translational Breast Cancer Research course taught here at BCM by faculty members of the Breast Center (December 2007)
- attended the San Antonio Breast Cancer Symposium (December 2007)
- presented data in poster format at the Molecular and Cellular Biology Department student symposium at BCM (April 2008)
- contributed a section to a text book chapter entitled "Insulin-Like Growth Factor Signaling in Normal Mammary Gland Development and Breast Cancer Progression," which was published earlier this year
- am primary author of a review article entitled "The Type-I Insulin-Like Growth Factor Receptor Pathway: A Key Player in Cancer Therapeutic Resistance"; this article was published earlier this year and largely focuses on the role of the insulin-like growth factor pathway in mediating resistance to numerous cancer therapies, including endocrine therapies, such as ICI (May 2008)
- attended and presented data in poster format at the DOD Era of Hope meeting (June 2008)
- am second author of a manuscript entitled "Insulin-Like Growth Factor-I Activates Gene Transcription Programs Strongly Associated with Poor Breast Cancer Prognosis," which was just published this past month (September 2008)

2) Research Project

Preliminary experiments performed in C4-12 cells (an ER-negative variant of MCF-7 cells that my mentor has shown can be reconstituted with a fully functional ER (1)) have shown that a cytoplasmic ER mutant (cER) that lacks a nuclear localization sequence (NLS) is completely resistant to ICI-mediated degradation. However, a rhodopsin-fused full-length ER (containing its NLS) that is sequestered at the plasma membrane is degraded upon treatment with ICI. **I hypothesize, therefore, that the NLS domain itself, and not merely the subcellular localization, is the critical determinant in ICI-mediated degradation of ER**.

Before presenting my findings of the past year (September 2007 – September 2008), I would first like to summarize the data from last year's progress report (covering September 2006-September 2007).

- cER, lacking the NLS domain, is resistant to ICI-mediated degradation following 12hr of treatment with ICI in MCF-7 cells; however, cER may start to be degraded by ICI after 24hr of treatment in MCF-7 cells; thus, the resistance to ICI-mediated degradation may be a temporal effect
- rhodopsin-fused full-length ER (RhER) containing its NLS and sequestered at the plasma membrane might be degraded by increasing concentrations of ICI in MCF-7 cells; however, these results are inconclusive since immunoblot detection of RhER is difficult

Over the past year, I have worked hard to address our concerns and move the project forward. Many of the issues regarding cER have been resolved. However, RhER protein levels have continued to be notoriously difficult to detect. Therefore, I have focused most of my attention on characterizing the ICI-mediated degradation of cER. Experiments involving RhER have been currently suspended, and, instead of using the rhodopsin-ER fusion protein, we have decided to use a different cloning strategy to examine whether the NLS domain itself or mere subcellular localization is important for ICI-mediated degradation of ER.

<u>Specific Aim 1:</u> Determine the role of nuclear localization, or the NLS, in ICI-mediated degradation of ER

• *Confirm that cER is capable of binding ICI*

Preliminary data shows that cER is resistant to ICI-mediated degradation. However, one concern of the project is that cER mutant receptor may be resistant to ICI merely because it is unable to bind ICI. I did not believe this to be the case for two reasons. First, the NLS deletion does not occur in the ligand-binding domain of the receptor. Second, cER is degraded by E2; thus, its ligand-binding ability is likely intact. However, to directly prove that the inability of ICI to degrade cER is not due to an inability of the cER mutant receptor to bind ICI, I needed to perform competitive binding assays (Fig. 1). I used C4-12 cells (MCF-7 derivatives that no longer express endogenous ER) stably expressing either GFP-wild-type ER (GFP-ER) or GFP-cER for the assay. The data obtained from these experiments proves that cER is capable of binding ICI with approximately the same affinity as wild-type ER. **Therefore, the inability of ICI to degrade cER is <u>not</u> due to an inability of the receptor to bind ligand.**

• *Confirm that cER is completely resistant to degradation by ICI in MCF-7 breast cancer cells*

Some of the data presented in the last progress report suggested that cER may start to be degraded by ICI if incubated in the presence of the ligand for a long enough period of time (24 hours). This observation was made in a single experiment and needed to be repeated. However, it raised the possibility that the resistance of cER to ICI may be affected by length of treatment. Along these lines, we also wondered if the phenotype could be influenced by increasing doses of ICI or altering culture conditions. These critical questions needed to be addressed to prove that cER is truly resistant to ICI-mediated degradation.

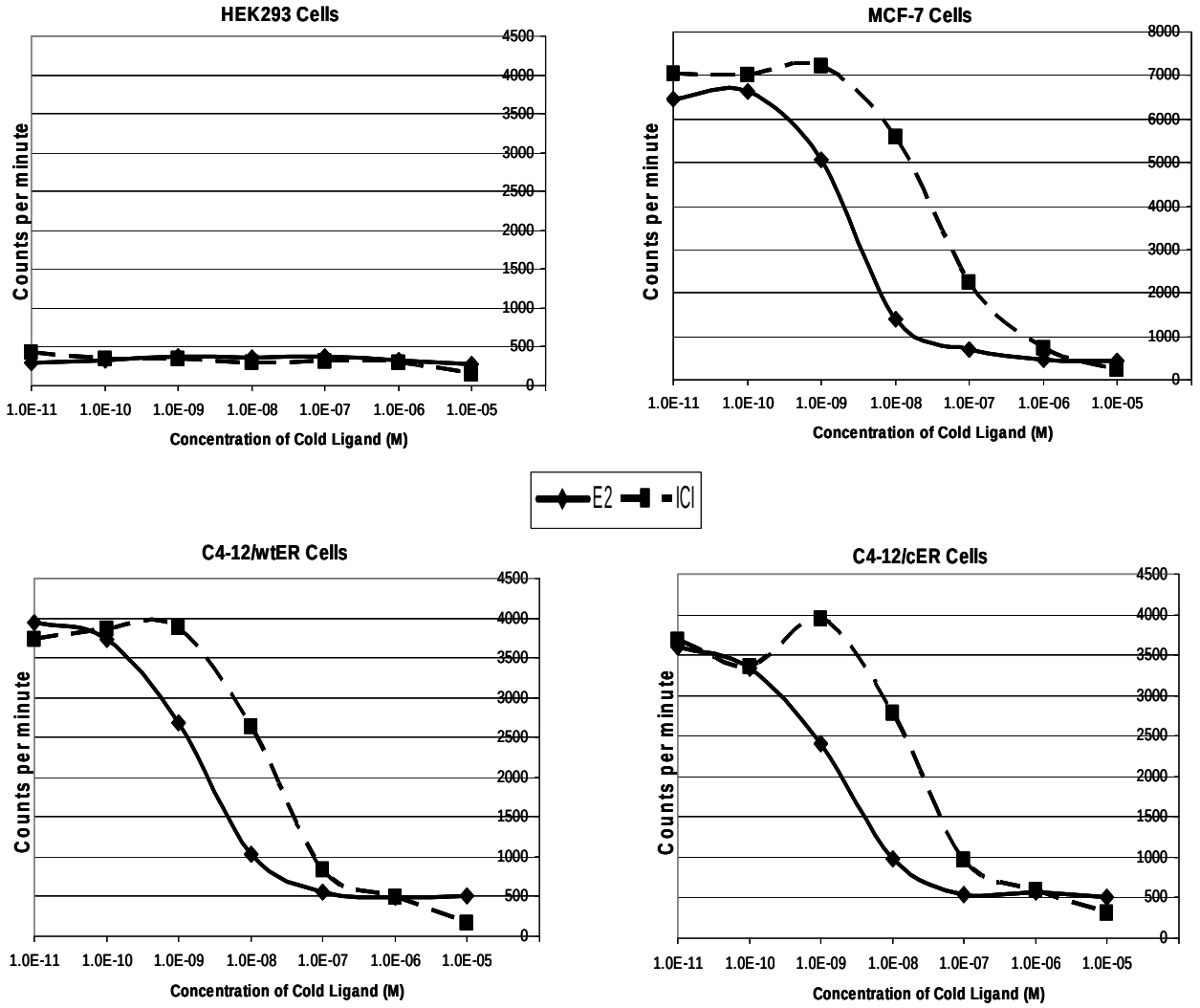

Figure 1. cER binds ICI with approximately the same affinity as wild-type ER. C4-12 cells stably expressing either GFP-wild-type ER (GFP-wtER) or GFP-cER were starved overnight in 5% charcoal-stripped serum (CSS). The next day, cells were incubated for 2hr at 37°C in CSS containing a fixed amount of tritiated estradiol (1.5 pmol) plus increasing concentrations of either nonradiolabeled E2 (10^{-5} M – 10^{-11} M) or nonradiolabeled ICI (10^{-5} M – 10^{-11} M). A scintillation counter was used to determine the amount of tritiated E2 bound to receptor. The same experiment was also performed in MCF-7 cells (ER-positive) and in HEK293 cells (ER-negative) as a positive and negative control, respectively. Solid line – nonradiolabeled E2; Dashed line – nonradiolabeled ICI

To determine if cER is resistant to ICI-mediated degradation over time, I used wild-type, ER-positive MCF-7 cells stably expressing GFP-cER (Fig. 2). As expected, endogenous ER is degraded upon treatment with either estradiol (E2) or ICI at both 6hr and 24hr. However, while cER is degraded by E2 at both time points, ICI is unable to degrade cER at either 6hr or 24hr. **Thus, cER is resistant to ICI-mediated degradation over time (up to 24hr) in MCF-7 cells.**

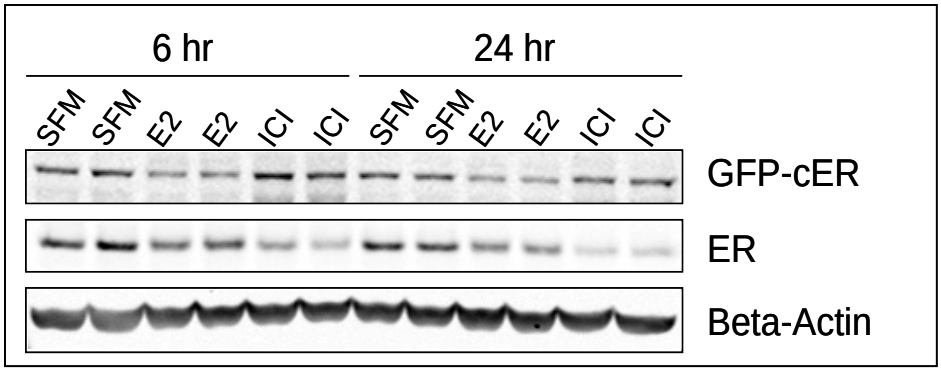

Figure 2. cER is resistant to ICI in MCF-7 cells over time. MCF-7 cells stably expressing cER were starved in serum-free medium (SFM) overnight and then either maintained in SFM or treated with E2 (10^{-9} M) or ICI (10^{-9} M) for either 6hr or 24hr. Protein levels were detected by Western blot using antibodies against either ER or beta-actin. Experiment was performed in duplicate.

We were concerned that cER might be degraded by ICI if the dose of ICI was sufficiently high. Therefore, to test this question, we used MCF-7 cells stably expressing GFP-cER (Fig. 3). As expected, endogenous ER is degraded upon treatment with increasing concentrations of either E2 or ICI. However, while cER is degraded by E2 in a dose-dependent manner, **ICI, even at a concentration of 10^{-7} M (100 times more concentrated than the highest dose previously tested), is unable to degrade cER.**

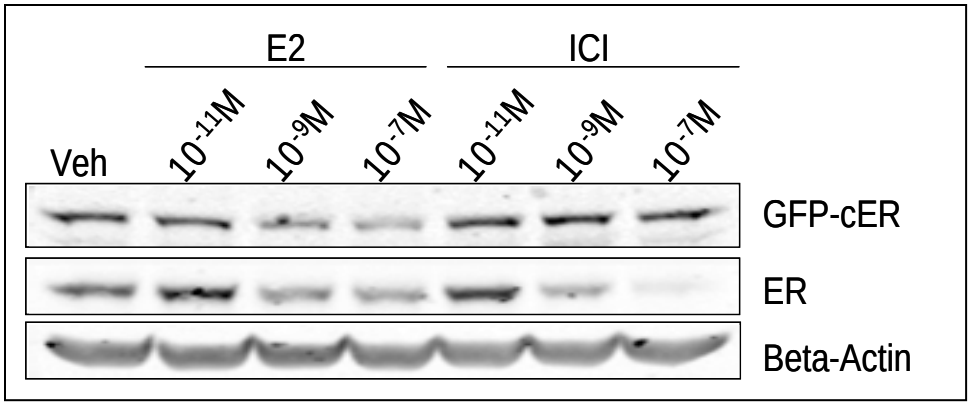

Figure 3. cER is resistant to ICI in MCF-7 cells treated with high concentrations of the ligand. MCF-7 cells stably expressing cER were starved in 5% charcoal-stripped serum (CSS) overnight and then treated for 8hr with vehicle (100% ethanol) or with increasing concentrations of either E2 (up to 10^{-7} M) or ICI (up to 10^{-7} M). Protein levels were detected by Western blot using antibodies against either ER or beta-actin. A similar experiment was performed in which treatments were maintained for 24hr; results were similar.

Many of my early experiments were conducted in serum-free medium (SFM). However, the majority of studies examining the effect of estrogen on cell culture are not conducted under strictly serum-free conditions. Instead, most studies are performed in medium containing charcoal-stripped serum (CSS). The charcoal stripping process is used to remove hormones from the serum while leaving many growth factors and other components essential for cell growth and survival in tact. Therefore, results obtained from cells cultured in CSS may be more physiologically relevant than data obtained from cells cultured under serum-free conditions. To

determine if cER is resistant to ICI-mediated degradation in cells cultured in CSS as it is in cells cultured in SFM, I used MCF-7 cells stably expressing GFP-cER (Fig. 4). As expected, endogenous ER is degraded by E2 and ICI at both time points (6hr and 24hr) and under both culture conditions (SFM and CSS). Furthermore, consistent with data obtained from cells cultured in SFM, cER expressed in MCF-7 cells cultured in CSS is degraded by E2 but resistant to ICI-mediated degradation. Based on these results, starvation of cells in CSS does not seem to affect the degradation phenotype. Since CSS is preferentially used over SFM by those in the field, my future experiments will be carried out in medium containing CSS.

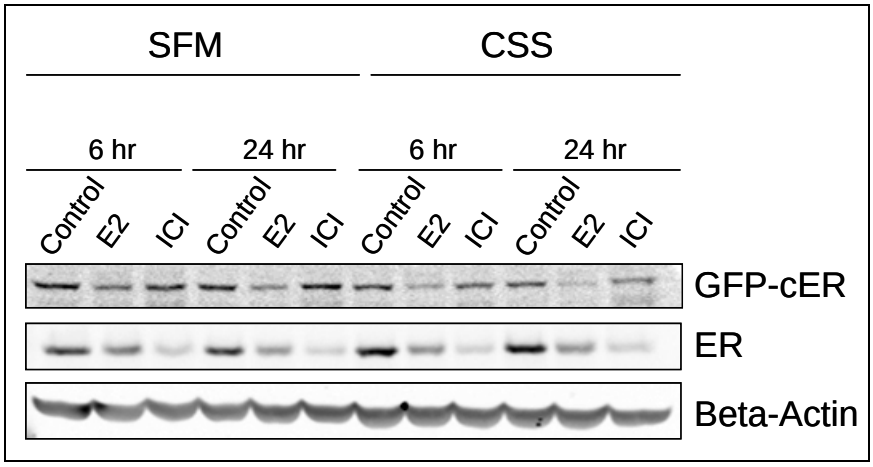

Figure 4. cER is resistant to ICI-mediated degradation in MCF-7 cells cultured in either SFM or CSS. MCF-7 cells stably expressing cER were starved in either serum-free medium (SFM) or medium containing 5% charcoal-stripped serum (CSS) overnight and then treated for 6hr or 24hr with E2 (10^{-9} M) or ICI (10^{-9} M). Protein levels were detected by Western blot using antibodies against either ER or beta-actin.

• *Characterize the role of the ER NLS in degradation*

To more completely understand the roles of subcellular localization and the NLS domain of ER in ICI-mediated degradation, I plan to force the cER mutant protein into the nucleus using the strong SV40 NLS (8 amino acids). I am currently in the process of cloning the SV40 NLS onto the amino-terminus of the cER protein and would like to briefly describe my strategy and progress to date. The coding sequence for cER has already been cloned into pEGFP vector from Clontech; this is the vector that was used to generate the stable cell lines that I have used for my experiments thus far. I have digested pEGFP/cER with *EcoRI* restriction enzyme, and my goal is to insert the SV40 NLS coding sequence into this site. For my insert, I have generated a double-stranded oligo consisting of 30 nucleotides; 24 nucleotides encode the SV40 NLS, and the remaining nucleotides were put in place to create artifical sticky ends that would be generated by digestion with *EcoRI*. I am currently in the process of trying to ligate the insert into the digested vector but have been unsuccessful to date. I would still like to test some different conditions before I move on to a new cloning strategy. However, if I continuously encounter difficulties with this approach, I may move to a PCR-based method.

Once I have successfully cloned the SV40 NLS onto cER, I plan to generate stable cell lines that I can use to perform my experiments. By treating cells expressing cER/SV40-NLS with ICI and monitoring ER degradation, I can establish whether it is the ER NLS domain itself or merely subcellular localization of ER that is the critical determinant of ICI-mediated

degradation. Additionally, I plan to further examine the role of the ER NLS by determining if it can act as a degron by conferring degradation to a heterologous protein such as actin or GFP; cloning to address this question has not yet been started.

<u>Specific Aim 2:</u> Identify and characterize proteins binding specifically to ICI-bound ER and, in particular, to the NLS domain

• *Determine if ICI promotes interaction between ER and keratin 18 and if this interaction is lost with cER mutant protein*

A recently published paper from the laboratory of Ken Nephew suggests that keratins 8 and 18 (K8/18) are important for ICI-mediated degradation of ER (2). The data shows that ER can interact with both K8 and K18 upon stimulation with ICI, and this interaction is not observed when cells are treated with E2. Therefore, this interaction seems to be uniquely elicited by ICI. Furthermore, K8 and K18 may be important for ICI-mediated degradation of ER since the ability of ICI to reduce ER levels is abrogated in cells with diminished levels of both K8 and K18. Based on these findings, we wanted to determine if the ICI-stimulated interaction between ER and K18 (we have not tested K8, since an antibody raised against this protein was not readily available to us) is lost in our cER construct, which has a deletion in the NLS domain.

I began by confirming the ICI-stimulated interaction between wild-type ER and K18. To address this question, I performed an immunoprecipitation experiment with endogenous ER and K18 from MCF-7 cells. The data shows that K18 interacts with endogenous ER in MCF-7 cells (Fig. 5). The interaction is potentially enhanced by treatment with ICI. However, the K18 blot from the pull-down with the control IgG antibody also shows a band, and this complicates interpretation of the data. This experiment needs to be repeated, but the preliminary data does appear promising.

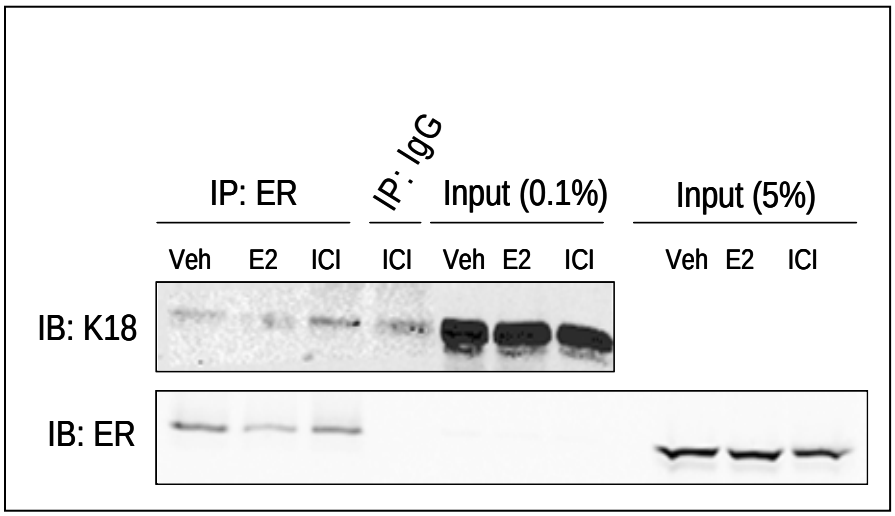

Figure 5. Interaction between ER and K18 may be enhanced upon stimulation with ICI. MCF-7 cells were starved in charcoal-stripped serum overnight and then stimulated with either vehicle (100% ethanol), estradiol (E2, 10^{-9} M), or ICI (10^{-9} M) for 1hr. Immunoprecipitation was performed with an ER antibody and protein G/sepharose beads. Immunoblotting was performed with an antibody against either K18 or ER.

Our original hypothesis is based on the idea that cER may be resistant to ICI because it lacks the NLS domain, which may serve as a region for interaction with other proteins that assist in the degradation process. Considering the Nephew lab's earlier findings, we wanted to determine if the ICI-stimulated interaction between ER and K18 is lost in our cER mutant construct. To address this question, I used C4-12 cells stably expressing either GFP-wild-type ER or GFP-cER (Fig. 6). The cells expressing wild-type ER were to be used as a control. However, the immunoprecipitation of ER from these cells was unsuccessful. This is likely due to the use of a lysis buffer that was too mild to disrupt the cell nucleus. I am currently in the process of optimizing conditions to perform successful immunoprecipitation from these cells. On the other hand, immunoprecipitation of ER from the C4-12/cER cells was successful (since ER in these cells is cytoplasmic, the nucleus does not need to be disrupted). A larger amount of ER was immunoprecipitated from the ICI-treated C4-12/cER cells. Therefore, it is impossible to say if the interaction between ER and K18 is increased by ICI treatment. However, it's probably a fair assumption that ER and K18 still interact even when the NLS domain is missing. **Therefore, it is unlikely that cER is resistant to ICI-mediated degradation because of an inability to interact with K18.** These experiments are being repeated to confirm this assumption.

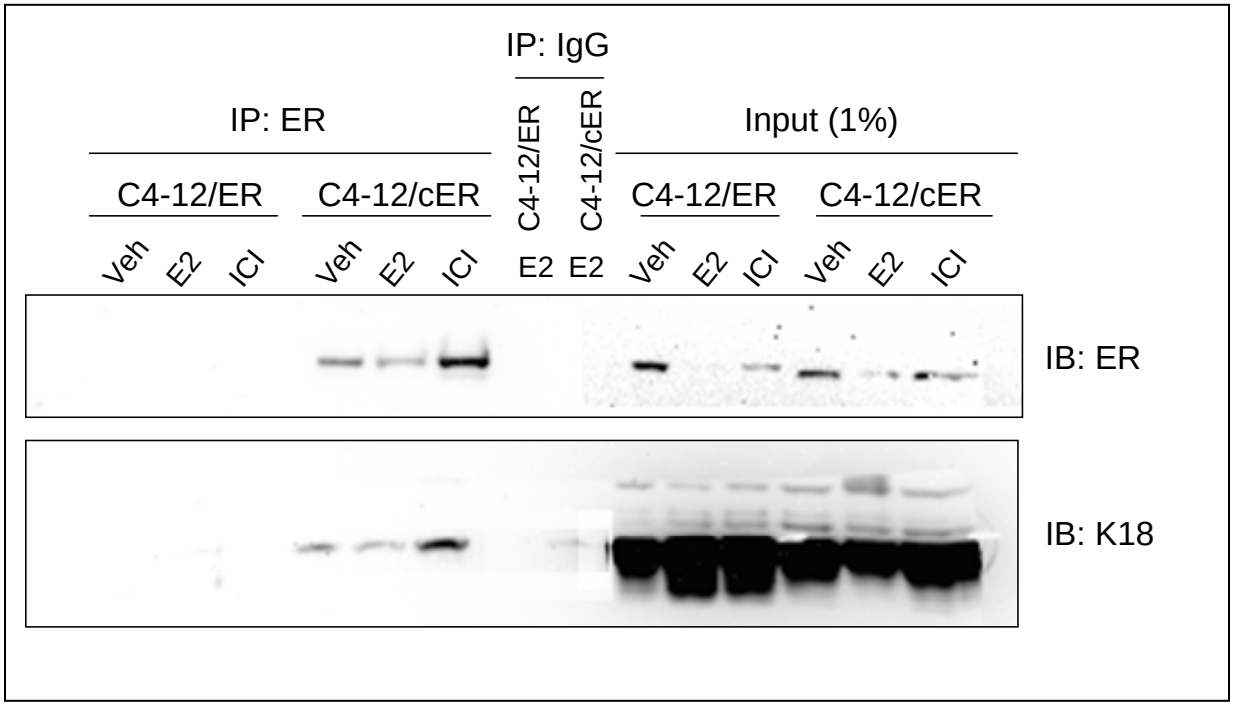

Figure 6. Interaction between ER and K18 probably does not occur via the NLS domain of ER. C4-12 cells stably expressing either GFP-wtER or GFP-cER were starved in charcoal-stripped serum overnight and then stimulated with either vehicle (100% ethanol), estradiol (E2, 10^{-9} M), or ICI (10^{-9} M) for 1hr. Immunoprecipitation was performed with an ER antibody and protein G/sepharose beads. Immunoblotting was performed with an antibody against either K18 or ER.

KEY RESEARCH ACCOMPLISHMENTS

- cER binds ICI with approximately the same affinity as wild-type ER; therefore, the inability of ICI to degrade cER is not due to an inability of the mutant receptor to bind ligand
- cER is completely resistant to ICI-mediated degradation in MCF-7 cells; this phenotype is independent of length of treatment (up to 24hr), concentration of ICI administered (up to 10^{-7} M), and culture conditions (serum-free medium vs. charcoal-stripped serum)
- Interaction between endogenous ER and K18 in MCF-7 cells may be enhanced upon stimulation with ICI
- Similar to full-length, wild-type ER, cER appears to maintain the ability to interact with K18; therefore, interaction between ER and K18 probably does not occur via the NLS domain of ER; furthermore, it is unlikely that cER is resistant to ICI-mediated degradation because of an inability to interact with K18

REPORTABLE OUTCOMES

- Casa, A., Litzenburger, B., Dearth, R., and Lee A.V. Insulin-like growth factor signaling in normal mammary gland development and breast cancer progression. Breast Cancer: Prognosis, Treatment, and Prevention. 2nd Ed. Editor: Jorge R. Pasqualini. New York, NY, 2008. 303-321.
- Casa, A., Dearth, R.K., Litzenburger, B.C., Lee, A.V., and Cui, X. The type I insulin-like growth factor receptor pathway: a key player in cancer therapeutic resistance. Front Biosci. 2008 May 1;13:3273-87. Review.
- Creighton, C.J., Casa, A., Lazard, Z., Huang, S., Tsimelzon, A., Hilsenbeck, S.G., Osborne, C.K., and Lee, A.V. Insulin-like growth factor-I activates gene transcription programs strongly associated with poor breast cancer prognosis. J Clin Oncol. 2008 Sep 1;26(25):4078-85.

CONCLUSION

ER is a critical marker for response to antiestrogen therapy. Although treatment with ICI is relatively successful, side-effects and the failure to respond in some patients is still a severe problem. In addition, indiscriminate degradation of ER results in the loss of its beneficial qualities in bone and the cardiovascular system. A better understanding of the mechanism by which ICI mediates ER degradation may lead to the development of new and better antiestrogen therapies to inhibit ER action with less toxicity and greater specificity.

The data I have presented to date confirms that cER is completely resistant to ICI-mediated degradation. This does not appear to be due to an inability of cER to interact with K18, which was previously suggested to be important for the ICI degradation process (2). I believe that the experiments I will perform over the next few months will be extremely informative. They will provide answers that will hopefully help answer whether it is the NLS domain or merely subcellular localization of ER that is the critical determinant of ICI-mediated degradation.

REFERENCES

1. Oesterreich, S., Zhang, P., Guler, R. L., Sun, X., Curran, E. M., Welshons, W. V., Osborne, C. K., and Lee, A. V. Re-expression of estrogen receptor alpha in estrogen receptor alpha-negative MCF-7 cells restores both estrogen and insulin-like growth factor-mediated signaling and growth. Cancer Res, *61:* 5771-5777, 2001.
2. Long, X. and Nephew, K. P. Fulvestrant (ICI 182,780)-dependent interacting proteins mediate immobilization and degradation of estrogen receptor-alpha. J Biol Chem, *281(14)*:9607-9615, 2006.

APPENDIX

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.

Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Angelo Casa	**Graduate Student**
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing,*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Stony Brook University, Stony Brook, NY	B.S.	2001-2005	Biochemistry
Baylor College of Medicine, Houston, TX	Ph.D. (in progress)	2005-present	Molecular Biology

Professional Experience

2003-2005 Undergraduate Research, Stony Brook University, Stony Brook, NY

Honors and Awards

2003 Golden Key International Honor Society, Stony Brook University, Stony Brook, NY

2005 Graduated Summa Cum Laude, Stony Brook University, Stony Brook, NY

2005 MCB Chair Scholar, Baylor College of Medicine, Houston, TX

2005 Rockwell Scholar, BRASS Organization, Baylor College of Medicine, Houston, TX

2007 Poster award winner at MCB Dept. Graduate Student Symposium, Baylor College of Medicine, Houston, TX

2007 Nominated to serve as a student representative on the Graduate Education Committee, Baylor College of Medicine, Houston, TX

Publications

1) Casa, A., Litzenburger, B., Dearth, R., and Lee A.V. Insulin-like growth factor signaling in normal mammary gland development and breast cancer progression. Breast Cancer: Prognosis, Treatment, and Prevention. 2nd Ed. Editor: Jorge R. Pasqualini. New York, NY, 2008. 303-321.
2) Casa, A., Dearth, R.K., Litzenburger, B.C., Lee, A.V., and Cui, X. The type I insulin-like growth factor receptor pathway: a key player in cancer therapeutic resistance. Front Biosci. 2008 May 1;13:3273-87. Review.

3) Creighton, C.J., Casa, A., Lazard, Z., Huang, S., Tsimelzon, A., Hilsenbeck, S.G., Osborne, C.K., and Lee, A.V. Insulin-like growth factor-I activates gene transcription programs strongly associated with poor breast cancer prognosis. J Clin Oncol. 2008 Sep 1;26(25):4078-85.